EPA Reviewer: Monique M. Perron, Sc.D **Signature**: _______________
Risk Assessment Branch I, Health Effects Division (7509P) Date: _______________
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Risk Assessment Branch I, Health Effects Division (7509P) Date: _______________

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DATA EVALUATION RECORD[1]

STUDY TYPE: Non-guideline mechanistic study

PC CODE: 129210 **DP BARCODE:** D432127

TEST MATERIAL (PURITY): Triflumezopyrim (99.4% a.i.)

SYNONYMS: DPX RAB55, 2,4-Dioxo-1-(5-pyrimidinylmethyl)-3-(3-(trifluoromethyl)-phenyl)-2H-pyrido(1,2-a)pyrimidinium inner salt

CITATION: Nabb, D.L. (2015); Triflumezopyrim (DPX-RAB55) technical: special design *in vitro* lung metabolism. DuPont Haskell Laboratory, Newark, Delaware, USA. Testing Facility Report No.: DuPont-44650. November 20, 2015. MRID 49382235.

SPONSOR: E.I. du Pont de Nemours and Company, Wilmington, Delaware 19898

EXECUTIVE SUMMARY:

The potential differential metabolism of triflumezopyrim in mouse and human lung tissue was assessed. Female human and female mouse lung microsomes were incubated with triflumezopyrim in the presence of an NADH-regenerating system and samples were collected over 180 minutes. Inhibitors specific to CYP2e1 (4-methyl pyrazole; 4MP) and CYP2f2 (5-phenyl-1-pentyne; 5PP) were added to the lung microsome assay to evaluate the specificity of both cytochrome P450 enzymes in the metabolism of triflumezopyrim.

The final reaction concentrations of 4MP, 5PP, and the test substance, triflumezopyrim, were 100 µM, 5 µM and 2.5 µM, respectively. All reactions were terminated by transferring 100 µL aliquots of the reaction mixture to 900 µL of acetonitrile over a 3 hour time period. Sample aliquots were removed at 6, 15, 30, 60, 90, 120 and 180 minutes. The heat-inactive microsomes were used as a negative control in the assay. Microsome extracts were analysed by ultra-high-performance liquid chromatography (UHPLC) coupled with tandem mass spectrometry (LC/MS/MS) by multiple ion monitoring (MRM).

Under the assay conditions, triflumezopyrim was not metabolized by female CD-1 mouse or female human lung microsomes with, or without inhibitors. The *in vitro* clearance rate of triflumezopyrim in female CD-1 mouse and female human microsomes could not be calculated and therefore, a comparison between species could not be obtained.

[1] This DER was generated by modifying the study summary in a Tier II document (MRID 49382105).

This non-guideline mechanistic study is adequate to aid in the evaluation of lung tumors observed in female mice in the mouse carcinogenicity study (MRID 49382174).

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	Triflumezopyrim technical
	Lot/Batch #:	RAB55-037
	Purity:	99.4%
	Description:	Solid
	CAS #:	1263133-33-0
	Stability of test compound:	Not reported
2.	Control materials	
	Negative (solvent) control:	100mM KH_2PO_4
	Inhibitor specific to CYP2e1:	4-methyl pyrazole (4MP)
	CAS #:	7554-65-6
	Lot #:	M0774
	Purity:	99.2%
	Supplier:	TCI America, Cambridge, Massachusetts, USA
	Inhibitor specific to CYP2f2	5-phenyl-1-pentyne (5PP)
	CAS #:	1823-14-9
	Lot #:	S13A011
	Purity:	99.0%
	Supplier:	Alfa Aesar, Ward Hill, Massachusetts, USA
3.	Species (Strain):	ICR/CD-1 mouse and human
	Vendor:	BioreclamationIVT, Baltimore, Maryland, USA (experimental microsomes) XenoTech, Lenexa, Kansas, USA (control microsomes)
	Sex:	Female
	Cell Type:	Lung microsomes, pooled (live reactions) Liver microsomes, pooled (heat-inactivated reactions)
	Protein Concentration:	Approximately 0.5 mg/mL mouse, 1.0 mg/mL human
	Reaction Buffer:	100 mM KH2PO4 with cofactors
	pH:	7.4
	Reaction Volume:	1.0 mL
	Dose Vehicle:	Acetonitrile
	Dose Volume	5 µL
	Dose Stock Concentration:	500 µM (199.17 ppm)
	Final Reaction Concentration:	2.5 µM (995.85 ppb)
	Replicates/Experiment:	3 test/species, 3 + inhibitor/species and 3 KH_2PO_4 buffer control
	Time Points:	6, 15, 30, 60, 90, 120, and 180 minutes
	Reaction Vessel:	20 mL Scintillation vial, loosely capped
	Incubation Temperature:	Approximately 37°C
	Extraction:	1:9, Sample: Acetonitrile
	Analytical Method:	LC/MS/MS

4. Microsome incubation sample preparation

Female ICR/CD-1 mouse and female human lung microsomes were stored at ≤-60°C until use. Microsomes incubations included either female mouse lung microsomes, female human lung microsomes or heat-inactivated mouse liver microsomes diluted in 100 mM KH_2PO_4 buffer (final protein concentration: approximately 1.0 mg/mL for human and 0.5 mg/mL for mouse, respectively). The diluted microsomes were incubated in the presence of a nicotinamide adenine dinucleotide (NADPH)-regenerating system (5 mM glucose-6-phosphate, 1 mM NADP, 2 mM $MgCl_2$, and 1 IU/mL glucose-6-phosphate dehydrogenase) with or without the addition of specific inhibitors, 4-methylpyrazole (4MP)(CYP2e1 inhibitor) or 5-phenyl-1-pentyne (5PP)(CYP2f2 inhibitor).

5. Concentrations and terminations

Concentrations

Concentration of triflumezopyrim at 2.5 μM was evaluated.

Termination times

Reactions were terminated at 6, 15, 30, 60, 90, 120, and 180 minutes.

B. STUDY DESIGN AND METHODS

1. Experimental start/completion
   11-June-2015 to 28-October-2015

2. Concentration verification

Although the stock solutions used to dose the incubations were not analysed, the initial time point (0 min) samples indicate that the test system was exposed to the approximate expected test substance concentrations (± 20% of expected target). Target concentration of test substance was 2.5 μM. The measured mean concentration of incubations dosed with triflumezopyrim at time 0 = 2.586 ± 0.043 μM. All dosing stock solutions were clear solutions and no precipitate was observed in the microsomal incubation system upon dosing.

3. Test conditions

The test reactions each had a final volume of 1 mL. The reactions containing the inhibitors were pre-incubated at 37°C for 10 minutes prior to the addition of 5 μL of inhibitor stock solution after which, the reactions were incubated an additional 15 minutes prior to the initiation of the reaction by the addition of 5 μL of test substance stock solution. The final reaction concentration of 4MP, 5PP, and triflumezopyrim was 100 μM, 5 μM and 2.5 μM, respectively. The reaction concentration of the test substance was chosen based on the sensitivity of the analytical method. The target reaction concentration (995 ng/mL) was approximately 100 times above the limit of quantitation (9.85 ng/mL) to allow for measurement of test substance depletion over time. The reaction concentration target was as low as analytically possible to avoid the risk of enzyme saturation. The reactions without inhibitors (including heat-inactivated and KH_2PO_4 buffer-only controls) were pre-incubated at 37°C for 10 minutes and the reactions were initiated by the addition of 5 μL of test substance stock solution. All reactions were terminated by transferring 100 μL aliquots of the reaction mixture to 900 μL of acetonitrile over a 3 hour time period. Sample aliquots were removed at 6, 15, 30, 60, 90, 120 and 180 minutes. Control liver microsomes were rendered inactive prior to the assay by diluting the microsomes in KH_2PO_4 buffer to 0.5 mg/mL and placing the capped tube in boiling water for 5 cycles. The heat-inactivated microsomes were used as a negative control in the assay.

4. Sample analysis and metabolic kinetics

Microsome extracts were analysed by ultra-high-performance liquid chromatography (UHPLC) coupled with tandem mass spectrometry (LC/MS/MS) by multiple ion monitoring (MRM). The calibration standards, fortification, and study samples were analysed by LC/MS/MS to quantify triflumezopyrim. The metabolic kinetics of triflumezopyrim were compared between mouse and human lung microsomes. To determine if triflumezopyrim metabolism was specific to CYP2f2 metabolism, the metabolic kinetics of triflumezopyrim was also compared in mouse and human microsome reactions in the presence of either CYP2e1 or CYP2f2 inhibitors.

II. RESULTS AND DISCUSSION

Triflumezopyrim eluted from the UHPLC column as a resolved peak at a retention time of approximately 1.7 minutes. The limit of quantitation (LOQ) for triflumezopyrim was calculated based on the lowest calibration standard concentration multiplied by the sample dilution factor of 10. The limit of detection (LOD) was defined to equal to 3/10 of the LOQ. Therefore, the LOQ and LOD for the quantitation of triflumezopyrim were 9.84 and 2.95 ng/mL, respectively.

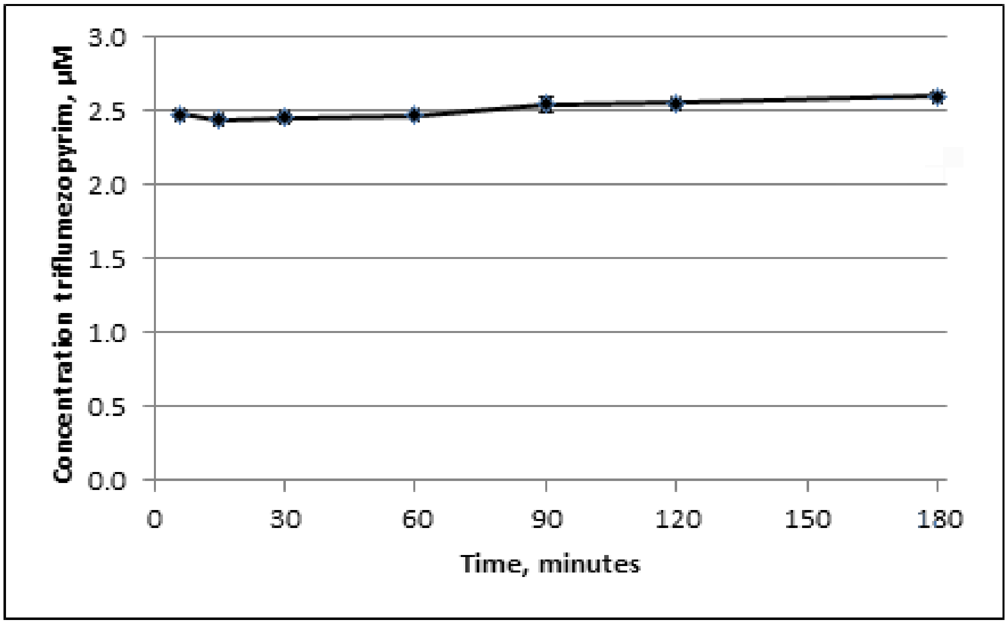
There was no measurable loss of triflumezopyrim observed under any of the test conditions; therefore, *in vitro* clearance rates could not be calculated. Although the reaction vials were loosely capped, a slight increase in test substance concentration was observed over the 3-hour incubation period (5–11%) in all reactions indicating that there was some limited evaporation from the test system over time.

Although a positive control substance was not included in this assay, it is assumed that the microsomes were functioning properly at the time of the assay. The vendor provided lot characterisation results indicating CYP450 activity in both the mouse and human microsomes prior to shipment. A later experiment, utilizing the purchased mouse and human lung microsomes from the same lots, indicated that CYP1A activity was 6.375 ± 0.775 and 4.649 ± 0.068 pmol resorufin/min/mg, in mouse and human microsomes, respectively, and CYP2B activity was 6.959 ± 0.441 and 1.563 ± 0.031 pmol resorufin/min/mg in mouse and human microsomes, respectively. These results provided additional confidence that the microsomes were functioning properly after shipment and storage at ≤-60°C.

The heat-inactivated microsome and KH_2PO_4 control reactions indicated that there was no loss of triflumezopyrim from the test system due to volatility or non-enzymatic loss such as hydrolysis under these test conditions.

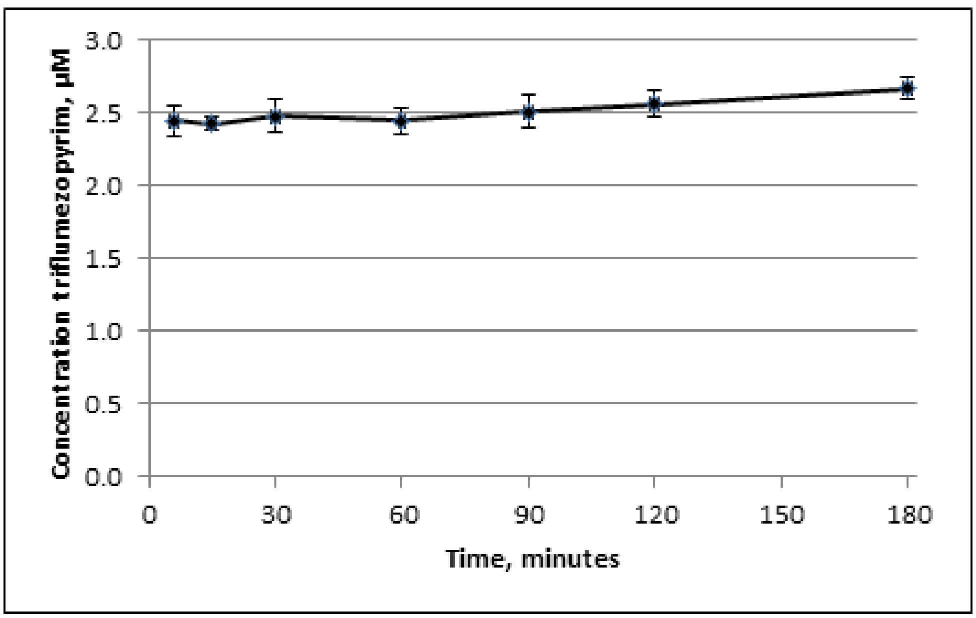
Figures 1 and 2 present the findings for triflumezopyrim exposures to mouse and human lung microsomes, respectively, without inhibitors. Similar findings were observed with inhibitors (pages 17-24, 37-39, and 41-45 of the study report).

Figure 1. Concentration of triflumezopyrim in live mouse lung microsome reactions



Taken from page 17 of the study report (MRID 49382235).

Figure 2. Concentration of triflumezopyrim in live human lung microsome reactions



Taken from page 18 of the study report (MRID 49382235).

III. CONCLUSION

A. <u>INVESTIGATOR'S CONCLUSIONS</u>:

Under the assay conditions, triflumezopyrim was not metabolized by female CD-1 mouse or human lung microsomes with or without inhibiters. The *in vitro* clearance rate of triflumezopyrim in female CD-1 mouse and human microsomes could not be calculated and therefore, a comparison between species could not be obtained.

B. <u>REVIEWER COMMENTS</u>:

The reviewers agree with the investigator's conclusions.

C. <u>STUDY DEFICIENCIES</u>:

None identified.